Nε-(carboxymethyl)lysine in debris from carotid artery stenting: multiple versus non-multiple postoperative lesions

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Running title: Nε-(carboxymethyl) lysine in carotid stenting
Abstract

**Background:** No predictor of postoperative ischemic events has been identified in patients undergoing carotid artery stenting. We aimed to determine whether Nε-(carboxymethyl)lysine in debris trapped by an embolic protection filter device is a predictor of postoperative ischemic events. **Methods:** We enrolled 27 patients (73.4 ± 7.2 years; 22 male, 5 female) who underwent carotid artery stenting for carotid artery stenosis. Diffusion-weighted magnetic resonance imaging was performed before and after the procedure. Protein samples were extracted from the debris. Nε-(carboxymethyl)lysine and myeloperoxidase were examined by solid phase enzyme-linked immunosorbent assay and western blot analysis.

**Results:** Seventeen patients had zero or one new lesion (non-multiple lesions) postoperatively, whereas 10 patients had two or more new lesions postoperatively (multiple lesions). All three patients with transient neurological deficits after angioplasty showed multiple lesions. The Nε-(carboxymethyl)lysine concentration of the protein sample was significantly higher in patients with multiple lesions than in those with non-multiple lesions (6.26 ± 2.77 ng/mg protein and 3.36 ± 1.57 ng/mg protein, respectively; \( P = 0.010 \)). Statin therapy for dyslipidemia was associated with a lower incidence of multiple lesions and a lower concentration of Nε-(carboxymethyl)lysine in the protein sample (\( P = 0.004 \) and \( P = 0.02 \), respectively). Receiver operating characteristic analysis showed that the area under the curve for Nε-(carboxymethyl)lysine was significantly greater than 0.5 (0.877, 95% confidence interval: 0.742–1.00). **Conclusions:** Nε-(carboxymethyl)lysine derived from debris may distinguish between patients with postoperative multiple ischemic lesions and those with postoperative non-multiple lesions who undergo carotid artery stenting.
**Introduction**

Carotid artery stenting (CAS) is widely used to treat severe carotid artery stenosis. However, the benefits of CAS depend on the risk of procedural neurological complications. A main complication of CAS is an ipsilateral cerebral embolism of the treated artery. A new ischemic lesion detected on diffusion-weighted magnetic resonance imaging (DWI), a potential forerunner of cerebral embolism, was observed in 10–40% of patients who underwent CAS (1-3). The characteristics of the carotid plaque are one of the predisposing factors for new DWI ischemic lesions after CAS. Various circulating biomarkers associated with inflammation and oxidative stress such as myeloperoxidase (MPO) have been implicated in the instability and rupture of carotid atherosclerotic plaque (4). Imaging techniques, including magnetic resonance imaging (MRI) and ultrasound, have been used to evaluate the characteristics of carotid artery plaque. However, no biomarker has yet been identified for predicting new postoperative DWI ischemic lesions after CAS.

Nε-(carboxymethyl)lysine (CML) is one of the advanced glycation end products generated by a non-enzymatic reaction with glucose and protein. These are elevated in patients with diabetes mellitus and dyslipidemia, and they accumulate in atheroma and foam cells in association with plaque progression (5,6). Serum CML levels have been reported to increase in non-diabetic patients who suffer from coronary heart disease (7). Statin therapy has been associated with a decrease in CML levels and embolic particles in the filter device after CAS (8,9). However, CML can be derived from another pathway related to inflammation. MPO can be expressed in neutrophils and activating macrophages, and it plays a role in CML formation at the sites of inflammation (10). Moreover, the CML concentration is increased in human carotid rupture-prone plaques (11). These facts suggest that CML may be a predictor of new postoperative DWI ischemic lesions after CAS.
The goals of the present study were 1) to identify CML and MPO in debris trapped by an embolic protection filter device during CAS; and (2) to determine whether these agents can predict postoperative ischemic events.

**Materials and Methods**

**Study Subjects**

We collected debris trapped by embolic protection filter devices in 27 consecutive patients (22 males, 5 females) who underwent CAS for severe carotid stenosis between August 2011 and February 2012 at Fukuoka University Chikushi Hospital. Patients who did not undergo MRI before CAS were excluded. The filter containing the debris (FilterWire EZ; Boston Scientific, Boston, MA, USA) was immediately washed with saline and stored at -40°C until use. Our study was approved by the research ethics committee of our institution (R11-033), and all patients gave informed consent.

**Clinical Data**

Patients’ characteristics, including age, sex, symptomatic or asymptomatic status, hypertension (blood pressure, ≥140/90 mmHg measured on repeated occasions or use of hypotensive drugs), diabetes mellitus (HbA1c, >6.5%; fasting blood glucose, >126 mg/dl; or use of insulin or oral glucose inhibitors), dyslipidemia (fasting serum low density lipoprotein, ≥140 mg/dl; triglyceride, ≥150 mg/dl; high density lipoprotein, <40 mg/dl; or use of statin therapy), new postoperative DWI ischemic lesions, and neurological deficits were recorded after the procedure. New lesions were confirmed by DWI and an apparent diffusion coefficient map and were then counted. The images were analyzed by two neurosurgeons who were not among the main operators or attending surgeons for CAS.
Carotid Artery Stenting Procedures

All patients were treated with CAS according to the North American Symptomatic Carotid Endarterectomy Trial criteria. The operative indication for CAS was defined as symptomatic carotid artery stenosis ≥50% or asymptomatic carotid artery stenosis ≥80%. Symptomatic patients underwent CAS following medication for 4 weeks after a cerebral ischemic attack. All patients took antiplatelet agents (aspirin, 100 mg daily; clopidogrel 75 mg daily; and cilostazol 200 mg daily) for at least 5 days preoperatively.

All CAS procedures were performed under general anesthesia. An 8-French sheath was introduced into the femoral artery, and heparin was injected into a vein until the activated clotting time exceeded 250 s (80 IU/kg). An 8-French guiding catheter was passed through the common carotid artery with the lesion, and after crossing the lesion, the FilterWire device was deployed distally into the internal carotid artery. Predilatation was performed with a 5–40 mm catheter balloon, and a 10–24 mm carotid stent was inserted in the internal to common carotid artery. Postdilatation was performed with a 7–20 mm catheter balloon. Antegrade blood flow was confirmed immediately by angiography.

Magnetic Resonance Imaging

New ischemic brain lesions were identified by comparing the DWI images acquired before the CAS procedure to those within 1–3 days after the procedure. DWI was performed using the Signa HDxt 1.5T Optima Edition MRI system (GE Healthcare, Milwaukee, WI, USA) with the following parameters: b-value, 1,000 s/mm²; repetition time/echo time/excitation: 5,000 ms/81.5 ms/l; matrix, 128 × 128; field of view, 240 × 240 mm; section thickness, 6 mm, interslice gap, 1.5 mm.

Protein Extraction

The protein was extracted from the debris trapped by the FilterWire. Briefly, the
FilterWire was placed in a 200-µL lysis buffer (0.1% Triton X-100 in PBS) at 4°C, and sonication was performed three times using a Bioruptor UCD-200TM (Tosho Denki Co., Ltd., Kanagawa, Japan) at 4°C for 10 min. The supernatant was collected after centrifugation (15,000 rpm for 15 min at 4°C), and the protein concentration was measured using the Bradford method (Quick Start Bradford protein assay kit; Bio-Rad Lab Inc., Hercules, CA, USA) with bovine serum albumin as standard.

**Western Blot**

Five microgram of each protein was applied to 10%-SDS PAGE, and immunoblotting was performed as previously described. Briefly, the protein was transferred to the nitrocellulose membrane and reacted with monoclonal anti-CML mouse antibody (1 µg/mL, 6D12) (12) or polyclonal anti-MPO rabbit antibody (1:4,000 dilution; Millipore Corp., Bedford, MA, USA) at 4°C for 12 h. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig)G (1:5,000 dilution; Medical & Biological Laboratories Co., Ltd., Aichi, Japan) or anti-rabbit IgG (1:5,000 dilution; Medical & Biological Laboratories Co., Ltd.) for 1 h. The signals were exposed on radiography films after chemiluminescence with Western Lightning (PerkinElmer Co., Ltd., Boston, MA, USA).

**Solid Phase Enzyme-linked Immunosorbent Assay**

The concentration of protein in each sample was adjusted to 2.5 µg/mL with carbonate-bicarbonate buffer, and each well of a 96-well microtiter plate was coated with 100 µL of each sample at 4°C overnight. After blocking with 0.5% gelatin/PBS solution, each well was incubated with 6D12 (1 µg/mL, 100 µL/well) or polyclonal anti-MPO rabbit antibody (1:4,000 dilution; 100 µL/well) for 1 h and was then reacted with HRP-conjugated anti-mouse IgG (1:5,000 dilution, Medical & Biological Laboratories Co., Ltd.) or
anti-rabbit IgG (1:5000 dilution, Medical & Biological Laboratories Co., Ltd.) for 1 h. The absorbance was read at 492 nm with a micro-enzyme-linked immunosorbent assay plate reader following chemiluminescence with hydrogen peroxide or o-phenylenediamine. The CML and MPO concentrations in each sample were calculated using a standard curve provided by CML-BSA (STA-314; Cell Biolabs, Inc., San Diego, CA, USA) and MPO (ab91116; Abcam Ltd., Cambridge, UK).

**Statistical Analysis**

Continuous variables were expressed as mean ± SD, and nominal variables were expressed as numerals and percentages. The Fisher’s probability exact test was used for comparisons of categorical data. Continuous variables were compared using Welch’s test. Relationships between two continuous variables were assessed using Pearson’s correlation analysis. A receiver operating characteristic (ROC) analysis was performed to determine whether CML and MPO can differentiate between patients with new multiple brain ischemic lesions and those with non-multiple brain ischemic lesions. Data analyses were performed using StatFlex, version 6.0 (Artech Co., Ltd., Osaka, Japan). A P-value <0.05 was considered statistically significant.

**Results**

**Patient Data and Clinical Results**

The patients were classified into two groups: those with non-multiple lesions (zero or one new postoperative cerebral ischemic lesion; n = 17) and those with multiple lesions (n = 10). Table 1 shows the clinical characteristics of patients in both groups. No significant difference in mean age was observed. Patients with multiple lesions had a higher incidence of symptomatic onset than those with non-multiple lesions (P = 0.043). No difference in the incidence of hypertension, diabetes mellitus, and dyslipidemia was found between the
groups. Neurologic events developed within a few days in three patients who had multiple lesions, which were categorized as reversible ischemic neurologic deficits.

**Western Blot Analysis**

Western blot analysis showed the presence of CML and MPO in the detergent extracts of the debris with protein bands of 63 kDa and 56/13 kDa, respectively (Figure 1). Both CML and MPO were detected in the debris of CAS samples 1 and 2, whereas the debris of CAS samples 4 and 5 showed an absence of CML and the presence of MPO, although the level of MPO was lower than that in CAS samples 1 and 2. The protein band of only CML was found in the debris of CAS sample 3.

**Solid Phase Enzyme-linked Immunosorbent Assay**

Immunoassay using antibodies against CML and MPO indicated that CML-BSA and MPO were bound to the solid phase in a concentration-dependent manner (data not shown). CML levels in debris were significantly higher in patients with multiple lesions than in those with non-multiple lesions (6.26 ± 2.77 ng/mg protein vs. 3.36 ± 1.57 ng/mg protein, respectively; \( P < 0.01 \); Figure 2), whereas the MPO levels did not differ between the two groups (4.02 ± 2.17 ng/mg protein vs. 3.88 ± 3.80 ng/mg protein, respectively; \( P = \text{not significant} \)).

**The Effects of Statin Therapy**

Only 16 of 22 patients with dyslipidemia underwent statin therapy. The incidence of multiple ischemic lesions after CAS was significantly lower in patients with dyslipidemia receiving statin therapy than in those not receiving such treatment (\( P = 0.004 \); Table 2). The CML levels in the debris were also significantly lower in the former than in the latter (3.20 ± 1.30 ng/mg protein vs. 5.65 ± 1.85 ng/mg protein, respectively; \( P = 0.02 \); Figure 3),
whereas the MPO levels did not differ between the groups.

**Correlation between Nε-(carboxymethyl)lysine and Myeloperoxidase in Debris**

As shown in Figure 4, a significant correlation was observed between the CML and MPO levels in the debris \( (r = 0.44; P = 0.022) \).

**Receiver Operating Curve Analysis**

Figure 5 shows the ROC analysis of CML and MPO for discriminating between patients with multiple lesions and those with non-multiple lesions. The ROC area under the curve (AUC) for CML was 0.877 (95% confidence interval [CI]: 0.742–1.00), whereas that for MPO was 0.627 (95% CI: 0.493–0.889).

**Discussion**

The present study demonstrates for the first time that 1) the concentrations of CML in the debris trapped by an embolic protection filter device were significantly higher in patients with postoperative multiple ischemic lesions than in those with postoperative non-multiple lesions; 2) the CML concentrations helped distinguish the patients with postoperative multiple ischemic lesions from those with non-multiple lesions; and 3) statin therapy for dyslipidemia decreased the incidence of multiple ischemic lesions and the concentration of CML in the debris.

Soft and unstable plaques are prone to the generation of postoperative ischemic lesions after CAS (13,14). In addition, an ulcerated carotid stenosis or a plaque length of >1 cm is an independent risk factor for new ipsilateral DWI lesions (15). An accumulation of CML, one of the major advanced glycation end products (AGEs), in an atherosclerotic lesion is associated with the progression of atheromatous plaque (10). Recently, CML levels were reported to increase in human rupture-prone carotid artery plaques and to mediate the
progression of stable to unstable plaques (11). Another report found no difference between the CML concentrations in the sera of symptomatic and asymptomatic patients with carotid artery stenosis (16). Semiquantitative analysis showed no significant difference between the AGEs in carotid plaque specimens from symptomatic and asymptomatic patients (16). In our study, the CML content was significantly higher in the debris from patients with multiple lesions than in the debris from those with non-multiple lesions. Additionally, ROC analysis for distinguishing between patients with multiple lesions and those with non-multiple lesions showed that the AUC for CML was significantly >0.5 (0.877, 95% CI: 0.742–1.00). Thus, CML in the debris distinguished between the patients who developed multiple cerebral ischemic lesions after CAS and those with non-multiple cerebral ischemic lesions.

According to DWI, CAS has been shown to cause cerebral micro-embolization in approximately half of all cases. Protected patients revealed a significantly lower incidence of postoperative ischemic lesions after CAS than in unprotected patients (2,17). Patients who developed a stroke after CAS showed more new DWI lesions than those who did not develop a stroke after CAS (2). However, the procedures during CAS may cause cerebral micro-embolization. For instance, the cleavage of plaque by the balloon vasodilatation during CAS can induce multiple atheromatous embolisms (18). In our study, postoperative multiple ischemic lesions were found in all three patients who had postoperative neurological deficits. The proportion of symptomatic patients was greater among those with multiple lesions than in those with non-multiple lesions. Finally, the CML debris content in patients with multiple lesions was higher than in those with non-multiple lesions and was positively correlated with the MPO content that was found in unstable carotid plaque. These findings suggest that multiple cerebral ischemic lesions may be induced by micro-embolization during and after CAS in patients with unstable carotid plaque.
AGEs can be derived from several pathways. Previous reports have demonstrated that glycated and/or oxidized low-density lipoprotein (LDL) accumulated in arterial intima contributes to the development of atherosclerosis (19,20). MPO is formed by neutrophils and activating macrophages, and contributes to the progression and destabilization of the plaque (21,22). Recently, MPO produced by neutrophils and activating macrophages has been shown to generate CML-modified proteins (23). Glycolaldehyde-pyridine, an MPO-derived advanced end product, is shown to accumulate in human atherosclerotic plaque (24). In this study, the concentration of CML in debris was positively correlated with that of MPO. Therefore, CML may be formed, not only by glycoxidation of LDL but also by MPO derived from inflammatory cells such as neutrophils and macrophages.

Recent studies showed the pleiotropic effects of statin therapy, including attenuation of vascular inflammation, improved endothelial cell function, and stabilization of plaque (25,26). It has been suggested that statin therapy may prevent receptor (R)AGE-mediated atherogenesis by reducing serum AGEs and increasing the soluble RAGE (27). Statin therapy is associated with smaller quantities of embolic particles in the filter device after CAS (9). The randomized ARMYDA-9 CAROTID study demonstrated that preoperative statin therapy significantly reduced the transient ischemic attack or stroke rate at 30 days and the generation of postoperative cerebral ischemic lesions after CAS (28). Our study showed that statin therapy was associated with a decreased incidence of multiple lesions after CAS. The CML content in the debris of patients receiving statin therapy was significantly lower than in those without statin therapy. These results suggest that statin therapy can prevent the generation of multiple cerebral ischemic lesions after CAS, as a result of the decreased CML formation.

This study has some limitations. Firstly, because of the small number of patients (27 cases), we cannot determine whether CML may be used to distinguish between patients
with multiple postoperative ischemic events and those with non-multiple ischemic events independent from other factors, such as diabetes mellitus, dyslipidemia, or soft and unstable carotid plaques. Secondly, we measured the CML content in debris trapped by the filter device. The quantity and quality of the trapped debris are dependent on the embolic protection device and the CAS procedure. Data on the serum levels of CML may be more useful for predicting postoperative ischemic events in patients with carotid artery stenosis.

Conclusions

CML derived from debris may help distinguish between patients with multiple postoperative ischemic events and those with non-multiple ischemic events who undergo CAS. However, the precise role and predictive value of CML needs to be confirmed by larger studies that include data on the serum CML levels.

Acknowledgments

The authors thank Miss Fukagawa for her technical assistance.

References


Figure Legends

**Figure 1.** Western blot analysis of \(N^ε\)-(carboxymethyl)lysine (CML)-modified protein and myeloperoxidase (MPO) in the debris.

The protein samples of debris are subjected to SDS-PAGE and western blot analysis using the 6D12 antibody and anti-MPO antibody. Panel A indicates CML-modified protein, which is expressed at about 63 kDa. Panel B indicates two bands of MPO (59 kDa and 13.5 kDa molecular weight molecules). The CML-modified protein and MPO are detected in CAS samples 1 and 2. In contrast, CAS samples 4 and 5 show the absence of CML-modified protein and the presence of MPO, though less than in samples 1 and 2. Only CML-modified protein is detected in sample 3.

**Figure 2.** Comparison of \(N^ε\)-(carboxymethyl)lysine (CML) and myeloperoxidase (MPO) contents in the debris of patients with multiple and non-multiple lesions.

The CML and MPO were measured by solid phase enzyme-linked immunosorbent assay with the monoclonal anti-CML antibody (6D12) and polyclonal anti-MPO antibody, respectively. Data are expressed as mean ± standard deviation. The CML content was significantly higher in patients with multiple lesions (6.26 ± 2.77 ng/mg protein, \(n = 10\)) than in those with non-multiple lesions (3.36 ± 1.57 ng/mg protein, \(n = 17\)). In contrast, the MPO content did not differ between patients with multiple and non-multiple lesions (4.02 ± 2.17 ng/mg protein vs. 3.88 ± 3.80 ng/mg protein, respectively). The differences were tested using Welch’s test. \(*P = 0.010.\)

**Figure 3.** The effect of statin therapy on \(N^ε\)-(carboxymethyl)lysine (CML) and myeloperoxidase (MPO) contents in the debris among patients with dyslipidemia.

The CML and MPO were measured by solid phase enzyme-linked immunosorbent assay with the monoclonal anti-CML antibody (6D12) and polyclonal anti-MPO antibody, respectively.
Data are expressed as mean ± standard deviation. The CML content was significantly lower in patients with statin therapy (3.20 ± 1.30 ng/mg protein, n = 16) than in those without (5.65 ± 1.85 ng/mg protein, n = 6). In contrast, no difference was found in the MPO content for those with and without statin therapy (3.25 ± 3.51 ng/mg protein vs. 4.54 ± 3.02 ng/mg protein, respectively). The differences were tested using Welch’s test. *P = 0.02.

**Figure 4.** Linear regression analysis of the relationship between Nε-(carboxymethyl)lysine (CML) and myeloperoxidase (MPO) in the debris.

The contents of CML and MPO were measured by solid phase enzyme-linked immunosorbent assay with the monoclonal anti-CML antibody (6D12) and anti-MPO antibody, respectively. The CML content was positively correlated with the MPO content. Linear regression analysis was performed using Pearson’s correlation analysis (correlation coefficient: 0.44; P = 0.022).

**Figure 5.** Comparison of Nε-(carboxymethyl)lysine (CML) and myeloperoxidase (MPO) in the debris according to the receiver operative curve (ROC) analysis.

The contents of CML and MPO were measured by solid phase enzyme-linked immunosorbent assay with the monoclonal anti-CML antibody (6D12) and anti-MPO antibody, respectively. The ROC curves of CML and MPO were generated to distinguish between the patients with multiple lesions and those with non-multiple lesions. The area under the curves for CML and MPO were 0.877 (95% confidence interval [CI], 0.742–1.000) and 0.627 (95% CI, 0.493–0.889), respectively.
Table 1. Clinical characteristics of patients according to diffusion weight imaging.

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<th>New DWI lesion after CAS</th>
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<tr>
<td></td>
<td>Non-multiple lesion</td>
<td>Multiple lesions</td>
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<td>P</td>
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<td>No. of patients (n = 27)</td>
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<td>10</td>
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<tr>
<td>Mean age</td>
<td>72.6 ± 6.7</td>
<td>74.8 ± 8.1</td>
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<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Male (n = 22)</td>
<td>12 (54.5%)</td>
<td>10 (45.5%)</td>
<td>0.08</td>
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<td>Female (n = 5)</td>
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<td>Asymptomatic (n = 20)</td>
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<td>5 (25%)</td>
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<td>5 (71.4%)</td>
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<td>1 (14.3%)</td>
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<td>11 (55%)</td>
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<td>7 (38.9%)</td>
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<td>3 (33.3%)</td>
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<td>Neurological deficits after CAS</td>
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<td>Yes (n = 3)</td>
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Data are mean values ± standard deviation or n (%). Statistical analyses were performed using Welch’s test and Fisher’s probability exact test.

Non-multiple lesion: No lesion or single lesion.

Multiple lesions: Two or more new lesions.

DWI: diffusion weight imaging; CAS: carotid artery stenting.
Table 2. The effect of statin therapy on new diffusion weight imaging lesions among patients with dyslipidemia.

<table>
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<th>Multiple lesions</th>
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<td>No (n = 6)</td>
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<td>Yes (n = 16)</td>
<td>14 (87.5%)</td>
<td>2 (12.5%)</td>
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Data are n (%). Statistical analyses were performed using Fisher’s probability exact.

Non-multiple lesion: No lesion or single lesion.

Multiple lesions: Two or more new lesions.

DWI: diffusion weight imaging; CAS: carotid artery stenting.
<table>
<thead>
<tr>
<th>Molecular Weight</th>
<th>CAS sample 1</th>
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<th>CAS sample 3</th>
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CML: 63kDa

MPO: 59kDa

MPO: 13.5kDa

Figure 1
Figure 2
Figure 3
Figure 4